BIOCHEMICAL PATHWAYS IN LEGUME ROOT NODULE NITROGEN FIXATION

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CONTENTS

I. INTRODUCTION

Recent investigations into various aspects of the biology and chemistry of legume root nodules have provided a number of facts which may be logically arranged to support an hypothesis relating the roles of the known components of this symbiotic system. Hitherto, reviews of legume root nodule nitrogen fixation have endeavored to present a complete coverage of literature pertaining to the subject. In this paper reference will be made only to literature that seems to the author to be relevant to the hypothesis which is being presented. The result is therefore not a review but a statement of the hypothesis, followed by a discussion in which the theme of the statement is developed.

Bergersen and Briggs (8) demonstrated that the nitrogen-fixing tissue of soybean nodules consisted of plant cells within which the bacteroids were arranged in groups, each group being enclosed in a membrane envelope. It is postulated that these envelopes and their contents are the ultimate nitrogen-fixing units. Figure 1 illustrates the proposed sequences of the known reactions of nodule nitrogen fixation and introduces some speculation about the unknown steps.

II. THE HYPOTHESIS

The primary reactions of the activation of molecular nitrogen and its reduction to ammonia occur in the membrane envelope; the activated[1] nitrogen is the ultimate acceptor in an electron transport chain which begins in the bacteroids. One of the links in this chain is hemoglobin, which lies in solution within the membrane envelope. The host supplies, as products of photosynthesis, carbon compounds which are partially oxidized by the bacteroids and serve as the source of electrons for the reduction of the activated nitrogen. The products of the incomplete oxidation of the substrates then serve as acceptors of ammonia in the production of amino acids by the bacteroids. These amino acids diffuse away and become available to the host plant.

[1] The term "activated" is used to denote the forms in which nitrogen exists between N_2 and the first really identified product, NH_3.

III. EVIDENCE FOR THE HYPOTHESIS

A. Membrane Envelope is Site of Primary Reactions of Nitrogen

Fractionation of excised soybean root nodules which had been exposed to atmospheres containing a large excess of isotopic nitrogen (N_2^{15}) showed that the isotopic excess in the acid-soluble portion of the membrane fraction reached a maximum in less than 15 min (7). The excess N^{15} in the acid-soluble portion of the soluble fraction rose for about 2 hr, and was less than that of the membrane fraction at 15 min. The bacteroids were not labeled at all after 2 hr. These findings give strong support to the view that the membrane envelopes are the site of the primary reactions of nodule nitrogen fixation and that these reactions are followed by the transfer of the newly fixed nitrogen to the soluble fraction. Further support is given to this interpretation by experiments in which it was shown

that nodules ceasing to function because of age had the same initial isotopic excess in the membrane fractions as young active nodules but that the transfer of nitrogen to the soluble fraction was impaired.

B. Hemoglobin Functions in Electron Transport

It has been established that the hemoglobin content of nodules and their capacity for nitrogen fixation are closely related (25). It has also been shown by Smith (23) that this pigment is in solution and is confined to the bacteroid-containing cells of the nodules. In this laboratory (author's *unpublished observations*), histochemical methods for the demonstration of hemoglobin have been found to delineate the membrane envelopes, and electron micrographs of thin sections of soybean nodules show diffuse electron-scattering material within these (8). From these observations it seems very likely that the pigment *in vivo* is located in solution between the bacteroids and the membrane envelopes.

Keilin and Smith (18) showed that it was unlikely that hemoglobin functioned as an oxidation-reduction catalyst in a mechanism of fixation involving hydroxylamine, as had been suggested by Virtanen and Laine (24). Smith (23) then showed that this pigment was not involved in the transport of oxygen for nodule respiration, since concentrations of carbon monoxide which completely inhibited nitrogen fixation and which were sufficient to convert all the pigment to CO-hemoglobin did not affect the respiration of whole nodules.

Hamilton, Shug, and Wilson (17) found that sonic extracts of soybean nodules showed shifts of absorption spectra corresponding to oxidation of the hemoglobin from the Fe^{++} to the Fe^{+++} state when the gas phase was changed from helium to nitrogen. This finding was confirmed by Bergersen and Wilson (9), who also showed that this effect was not due to undetected traces of oxygen in the gas mixtures. In addition it was shown that bacteroids could reduce the oxidized form of hemoglobin in the absence of air, as had been previously observed by Appleby and Bergersen (2) when studying nodule homogenates with the hand spectroscope. The suggestion has therefore been made (9) that hemoglobin may function in legume root nodules in a cycle in which it is reduced by the bacteroids and oxidized

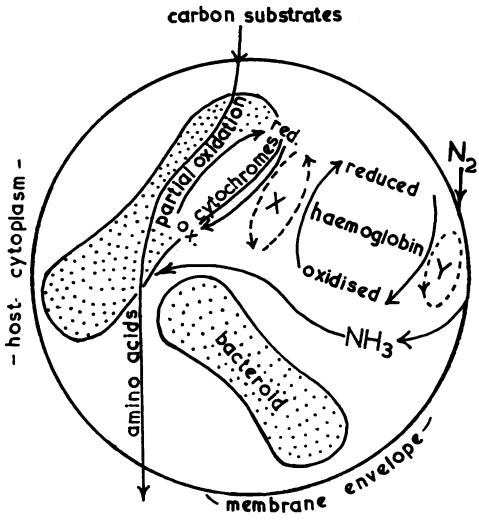

Figure 1. A diagramatic representation of the hypothesis. *X* represents unknown links between bacteroid cytochromes and hemoglobin that allow reduction of the pigment in the absence of air. *Y* represents the unknown links between hemoglobin and the site of conversion of molecular nitrogen to ammonia.

as a result of the processes of nitrogen fixation. That is, the sonic treatment of Hamilton *et al.* (17) produced fragments of the nitrogen-fixing system capable of a low order of activity, which was expressed as oxidation of the hemoglobin when the reducing source, the bacteroids, had been removed by centrifugation. Keilin and Smith (18) failed to see any spectroscopic evidence of the oxidized form of hemoglobin in whole nodules or in slices but this fact does not preclude its existence, masked by a great excess of the reduced form.

C. Other Links in Electron Transport Chain

The nature of the links between the bacteroid respiratory pathways and hemoglobin and between hemoglobin and the activated nitrogen at the fixing site are not known, but it is pertinent to mention the carbon monoxide-binding pigment found in bacteroids by Appleby (15). This pigment may possibly function as a link between the cytochromes and hemoglobin. An alternative possibility is that there is direct electron transfer between a cytochrome and hemoglobin in solution, since most of the bacteroid cytochromes are associated with the bacteroid cell membrane (15).

The results of Schneider, Hoch, and Burris

(21) show that there are apparently two phases of nodule metabolism concerned with hydrogen. First, hydrogen evolution occurs only in the absence of nitrogen; that is, it appears to be a release of metabolic hydrogen in the absence of the acceptor. Second, the exchange reaction between hydrogen and molecular deuterium requires the presence of nitrogen and is more sensitive to carbon monoxide than is hydrogen evolution. It may be that hydrogen evolution occurs at some step in the electron transport chain prior to hemoglobin, whereas the exchange reaction takes place at some stage of the reduction of nitrogen. Winfield (29) has suggested that hydrogenase may not have the same form in all organisms and may sometimes be an integral part of an enzyme known by another name; its principal role may be that of hydrogen transference and not the uptake or evolution of hydrogen. Hydrogen transfer may be the natural function of hydrogenases in nodules.

D. Oxygen Tension in Nodules

The hypothesis that hemoglobin has a role in electron transport requires that oxygen tension in the nitrogen-fixing units be very low indeed, since bacteroids isolated from crushed nodules have maximum Q_{O_2} at 0.02 atm of oxygen (11). Oxygen, if present at even quite low partial pressures, would certainly compete with hemoglobin for the terminal respiratory pathway of the bacteroids. However, the high respiratory rates of bacteroids in air and the fact that they are enclosed in the envelopes must mean that the oxygen tension in their immediate vicinity is only a fraction of that in the host cell, which would in turn be only a fraction of that in the root medium. Allison *et al.* (1) concluded that conditions within nodules were largely anaerobic when they found that respiration of intact nodules increased by more than 100 per cent when air was replaced by pure oxygen, whereas root respiration was unaffected. Ferguson and Bond (16), however, showed that nodulated plants required higher oxygen tensions in the root-growth medium than plants grown on combined nitrogen. A similar effect was found by Burris, Magee, and Bach (13); nitrogen fixation by excised soybean nodules increased as oxygen tension increased up to 0.5 atm, above which it declined sharply. Parker and Scutt (19) have shown that oxygen is a competitive inhibitor of nitrogen fixation in *Azotobacter*. A similar situation probably exists in nodules when oxygen tension begins to rise in the envelopes and oxygen begins to compete with hemoglobin for the terminal respiration of the bacteroids. The stimulation of fixation by oxygen tensions above that of air but below 0.5 atm, may be an expression of some auxiliary process.

E. Amino Acids Are Produced by Bacteroids

Photosynthetic products have been shown to be necessary for nitrogen fixation in nodules (26). $C^{14}O_2$ supplied to leaves of illuminated soybeans was rapidly translocated to the nodules, where the C^{14} appeared in amino acids. The evidence indicates that specific photosynthetic products, possibly of carbohydrate nature, are necessary substrates for nitrogen fixation in legume root nodules (4).

Bacteroids isolated from soybean root nodules oxidize supplied substrates only partially (6). This result may be either an expression of oxidation and assimilation, as shown for glucose by Burris and Wilson (12), or a block in metabolism at some subsequent step in substrate oxidation. Oxidation of substrates maintains the reduced state of the electron transport chain and the products of the incomplete oxidation may be used in the formation of amino acids.

Ammonia is accepted as being at the end of the nitrogen fixation pathways and the beginning of the assimilatory pathways (10, 20). Ellfolk and Katanuma (14) showed that soybean bacteroids had a high content of an ammonia-activating enzyme which functioned in amination reactions. This observation is in accord with the finding (Bergersen, *unpublished data*) that when soybean bacteroids were shaken with succinate, which they oxidize only to the extent of about 30 per cent (6), and 0.01 M ammonium sulfate, amino acids appeared quite rapidly in the medium.

Thus the products of photosynthesis supplied to the bacteroids as substrates may be envisaged as providing both the reducing source for the ultimate production of ammonia from molecular nitrogen and also the ammonia acceptors which the bacteroids use in the formation of the amino acids. These become available for host plant nutrition (3, 5, 22, 27, 28).

The hypothesis which has been presented is regarded by the author as a framework upon which future work may be based and it is fully

recognized that many gaps in knowledge must be filled before the validity of the suggested pathways can be tested.

IV. Summary

A working hypothesis of the function of various known components of legume root nodules in processes connected with nitrogen fixation is presented. The over-all reactions are seen as intimately connected functions of components of both plant and nodule bacteria, which are linked by an electron transport chain involving hemoglobin.

V. References

1. Allison, F. E., Ludwig, C. A., Hoover, S. R., and Minor, F. W. 1940 Biochemical nitrogen fixation studies. I. Evidence for limited oxygen supply within the nodule. Botan. Gaz., **101**, 513–533.
2. Appleby, C. A. and Bergersen, F. J. 1958 Cytochromes of *Rhizobium*. Nature, **182**, 1174.
3. Aprison, M. H., Magee, W. E., and Burris, R. H. 1954 Nitrogen fixation by excised soybean root nodules. J. Biol. Chem., **208**, 29–39.
4. Bach, M. K., Magee, W. E., and Burris, R. H. 1958 Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. Plant Physiol., **33**, 118–124.
5. Bathurst, N. O. 1954 Soluble constituents of lupin nodules. J. Exptl. Botany, **5**, 257–262.
6. Bergersen, F. J. 1958 The bacterial component of soybean root nodules; changes in respiratory activity, cell dry weight and nucleic acid content with increasing nodule age. J. Gen. Microbiol., **19**, 312–323.
7. Bergersen, F. J. 1960 Incorporation of N^{15} into various fractions of soybean root nodules. J. Gen Microbiol., **22**, 672–678.
8. Bergersen, F. J. and Briggs, M. J. 1958 Studies on the bacterial component of soybean root nodules; cytology and organization in the host tissue. J. Gen. Microbiol., **19**, 482–490.
9. Bergersen, F. J. and Wilson, P. W. 1959 Further spectrophotometric studies of soybean nodule extracts. Proc. Natl. Acad. Sci. U. S., **45**, 1641–1646.
10. Burris, R. H. 1956 Nitrogen fixation. In *Atomic energy commission report*, No. TID-7512, pp. 361–369. U. S. Government Printing Office, Washington.
11. Burris, R. H. and Wilson, P. W. 1939 Respiratory enzyme systems and symbiotic nitrogen fixation. Cold Spring Harbor Symposia Quant. Biol., **7**, 349–361.
12. Burris, R. H. and Wilson, P. W. 1942 Oxidation and assimilation of glucose by root nodule bacteria. J. Cellular Comp. Physiol., **19**, 361–371.
13. Burris, R. H., Magee, W. A., and Bach, M. K. 1955 The pN_2 and the pO_2 function for nitrogen fixation by excised soybean nodules. In *Biochemistry of nitrogen*, pp. 190–199. Suomalainen Tiedeakatemia, Helsinki.
14. Ellfolk, N. and Katanuma, N. 1959 The occurrence of ammonia activating enzyme in various organisms. Arch. Biochem. Biophys., **81**, 521–522.
15. Falk, J. E., Appleby, C. A., and Porra, R. J. 1959 The nature, function and biosynthesis of the haem compounds and porphyrins of legume root nodules. Symposia Soc. Exptl. Biol., No. **13**, 73–86.
16. Ferguson, T. P. and Bond, G. 1954 Symbiosis of leguminous plants and nodule bacteria. V. The growth of red clover at different oxygen tensions. Ann. Botany, **18**, 385–396.
17. Hamilton, P. B., Shug, A. L., and Wilson, P. W. 1957 Spectrophotometric examination of hydrogenase and nitrogenase in soybean nodules and Azotobacter. Proc. Natl. Acad. Sci. U. S. **43**, 297–304.
18. Keilin, D. and Smith, J. D. 1947 Hemoglobin and nitrogen fixation in the root nodules of leguminous plants. Nature, **159**, 692.
19. Parker, C. A. and Scutt, P. B. 1958 Competitive inhibition of nitrogen fixation by oxygen. Biochim. et Biophys. Acta, **29**, 662.
20. Roberts, E. C. 1959 Some observations on the chemistry of biological nitrogen fixation. Symposia Soc. Exptl. Biol., No. **13**, 24–41.
21. Schneider, K. C., Hoch, G. E., and Burris, R. H. 1959 Hydrogen metabolism of soybean root nodules. Federation Proc., **18**, 318.
22. Sen, S. P. and Burma, D. P. 1953 A study with paper chromatography of the amino acids in legume nodules. Botan. Gaz., **115**, 185–190.
23. Smith, J. D. 1949 Hemoglobin and the oxygen uptake of leguminous root nodules. Biochem. J., **44**, 591–598.
24. Virtanen, A. I. and Laine, T. 1946 Red, brown and green pigments in leguminous root nodules. Nature, **157**, 25–26.

25. VIRTANEN, A. I. 1955 Biological nitrogen fixation. Proc. Intern. Congr. Biochem., (Brussels), 3rd Congr., 425.
26. VIRTANEN, A. I., MOISIO, T., AND BURRIS, R. H. 1955 Fixation of nitrogen by nodules excised from illuminated and darkened pea plants. Acta Chem. Scand., **9**, 184–186.
27. WEINGRA, K. T. AND BAKHUIS, J. A. 1957 Chromatography as a means of selecting effective strains of rhizobia. Plant and Soil, **8**, 254–262.
28. WILSON, P. W. AND UMBREIT, W. W. 1937 Fixation and transfer of nitrogen in the soybean. Zentr. Bakteriol. Parasitenk., **96**, 402–411.
29. WINFIELD, M. E. 1955 Reactions of hydrogen gas in solution. Revs. Pure and Appl. Chem., **5**, 217–246.